

# **Using Progesterone as an Indicator of Ovarian Response to Stimulation in Cattle**

A Senior Project  
presented to  
the Faculty of the Animal Science Department  
California Polytechnic State University, San Luis Obispo

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by

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## Introduction

Fertility in dairy cows has been declining as herds become increasingly more productive. This decline is due to a number of biological reasons including, rapidly changing genetics correlated with selecting for productivity rather than for reproductive traits. This has resulted in cows failing to ovulate good quality oocytes, as well as a decrease in uterine receptivity. Shortcomings in herd management practices, including estrus detection and nutritional management have also contributed to this decline [1–3]. To increase fertility, knowledge of ovarian dynamics has been used to manipulate ovulation cycles in several ways: timed artificial insemination, superovulation and oocyte pick up (OPU) combined with in vitro embryo production, and embryo transfer [4]. Assisted reproductive technologies have become an increasingly prevalent way to combat the decreased fertility rates, shorten the generation interval, and concentrate genes from genetically valuable individuals [4–6]. The age at first calving is ideally just under two years old, which means that genetically valuable heifers are not spreading their genetics for years [2]. With the use of OPU, these genetically valuable animals of any age and virtually any reproductive status can produce offspring [4]. Furthermore, a cow is only capable of producing a limited number of offspring in her lifetime without the use of assistive reproduction technologies. To increase the number of offspring a cow can have, ovarian stimulation and embryo transfer can be employed.

Many techniques have been proposed to increase the number, as well as the quality of oocytes and embryos retrieved from cows undergoing ovarian stimulation. Despite this, rates have not changed significantly since the start of ovarian stimulation and OPU [6,7]. A major factor in this is the high variability between the individual responses of each cow. The variability in response to stimulation has been attributed to the quantity of immature oocytes in the ovarian reserves and varying levels of hormones during oocyte maturation [8,9]. Subsequently, the stimulation protocol has been associated with a decrease in oocyte quality. This has been observed as comparatively low blastocyst and pregnancy rates [7].

Numerous studies have been done on the various steroid hormones that are present during normal follicular waves and how they can be given exogenously to manipulate the ovarian wave dynamics. The one of interest to us is progesterone, a steroid hormone secreted by the corpus luteum (CL) and placenta. Progesterone can be given exogenously using a controlled internal releasing device (CIDR) inserted into the vagina. The circulating level of progesterone has been directly linked to circulating estradiol levels and inversely linked to luteinizing hormone (LH) pulse frequency [10]. Under a high concentration of progesterone, the decreased LH pulse frequency inhibits selection of the dominant follicle and prevents the LH surge that would cause ovulation [11,12].

Since the 1980s, low progesterone levels have been linked to a fewer number and lower quality of embryos produced in cows undergoing ovarian stimulation [13,14]. More recently, large meta analysis studies in humans have found that an increased progesterone concentration is associated with an increased ovarian response [15–17]. However, no significant difference in oocyte or embryo quality has been found in humans with varying progesterone levels [18,19]. This correlation between progesterone concentration, the number of follicles detected, and oocytes retrieved is likely due to the progesterone produced by the follicles during folliculogenesis in humans who, unlike cattle, do not have a CL present at the time of stimulation [20]. Dissimilar to the human protocol, cattle are given exogenous progesterone during ovarian stimulation. In cattle, CIDRs are used to supply a minimal dose of progesterone, which has been

shown to increase embryo production by regulating LH pulsatility [21]. A high progesterone concentration at the time of OPU has been associated with an increase in oocyte number as well as higher quality embryos in dairy cattle [8,9]. Nasser et. al. [8] and Rivera et. al. [9] found that using one or two CIDRs respectively minimized the adverse effects of low endogenous progesterone levels. Both studies also found an increase in number and quality of oocytes retrieved and embryos produced, which was theorized to be due to oocyte maturity at the time of fertilization [8,9]. However, contradictory results were found in a different study done on oocytes developed under high progesterone concentrations, which resulted in comparatively fewer embryos [22]. This study suggested that, a low level of progesterone during the growing phase after follicle stimulating hormone (FSH) stimulation, may mimic dominance in multiple follicles and result in an increased number of competent embryos [12,22].

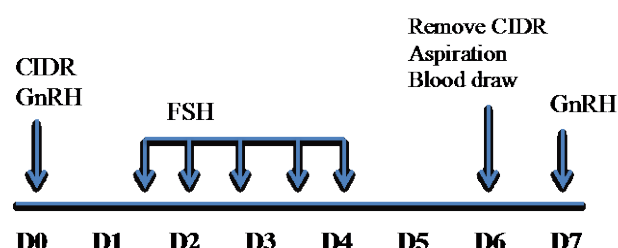
The objective of our study was to correlate circulating progesterone levels with the outcome of ovarian stimulation and OPU. To do this, we examined the relationship between progesterone levels at the time of follicular aspiration to the subsequent follicular number, oocyte quality, and blastocyst rates. The expected outcome was that a higher quantity and quality of embryos would be seen in animals with higher circulating progesterone levels at the time of OPU.

## Materials and Methods

### *Ovarian stimulation and collection*

Cows and heifers undergoing clinical OPU due to their high genetic merit were used. The animals were: 3 multiparous and 1 heifer Holstein, 1 multiparous and 8 heifer Jerseys, and 1 multiparous Angus. Each animal was exposed to the same protocol, though differing doses and FSH analogs were used. The protocol followed was adapted from the FSH coasting protocol in Nivet et. al. [23]. On day 0, each animal was given 2mL of GnRH (Cystorelin®, Meril®, Duluth, GA, USA) and a CIDR was placed (1.38g progesterone. Eazi-Breed™ CIDR® Zoetis). 36 hours later the cows began 10-20mg FSH (Follitrophin-V, Bioniche Animal Health, Belleville, ON, Canada; Pluset® Original, Minitube of America) injections every 12 hours for 2.5 days. A FSH coasting period of  $50 \pm 3$  hours passed and then the CIDR was removed on day 6 (Figure 1). At this time, the number and size of all follicles (diameter), corpus lutea (diameter by diameter), and cysts (diameter by diameter) were visualized using transvaginal ultrasonography performed by the same veterinarian at each OPU. All follicles were aspirated using transvaginal puncture with an 18G needle and aspiration unit (Minitube of America, Vernona, WI, USA). The follicular fluid was collected in 50 ml tubes containing  $15 \pm 5$ ml of warm HEPES-buffered Tyrode's medium (TLH) with Heparin. Corpus lutea were left intact and cysts were drained into separate collection tubes than the follicular fluid.

**Figure 1.** Ovarian Stimulation Protocol



### ***Progesterone analysis***

Blood was drawn from the caudal tail vein or jugular vein at the time of CIDR removal and OPU. The collected blood was allowed to coagulate before being centrifuged so plasma could be collected. All plasma samples were frozen until the progesterone assay was performed. The progesterone assays were performed by the Cornell Animal Health Diagnostic Center Endocrinology Laboratory (240 Farrier Road, Veterinary College, Cornell University, Ithaca, New York).

### ***In vitro maturation and fertilization***

The fertilization procedure used in Nivet et. al. [23] was followed. The collected follicular fluid was filtered to find oocytes, which were then washed to remove follicular fluid. The oocytes were immersed in maturation media and incubated for 24 hours at 39.5°C with 5% CO<sub>2</sub> in maximum humidity.

After maturation, oocytes were washed and transferred to oil droplets. The semen used was thawed and evaluated individually prior to fertilization then added to the droplets. The semen used was individually selected for each cow. The fertilization medium containing oocytes and spermatozoa was then incubated for 15-18 hours at 38.5°C with 5% CO<sub>2</sub> in maximum humidity.

The droplets were observed for embryo cleavage (>2 cells) 72 hours after fertilization. On day 6 after fertilization embryos were evaluated and prepared for shipment or freezing. Embryos scoring a 1 or 2 were recorded as good quality.

### ***Statistical analysis***

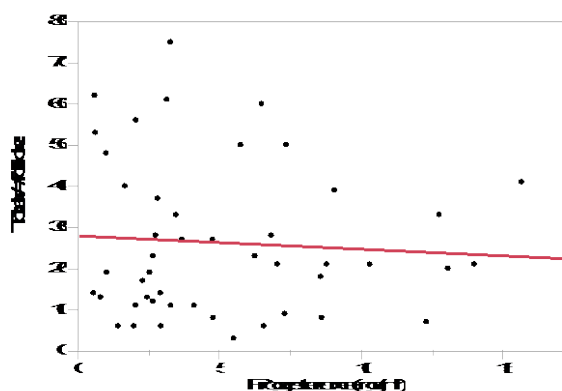
Statistical analysis was done using JMP Software (SAS Institute, SAS Campus Diveway, Cary, North Carolina 27513). The effect of progesterone on each response variable was done using a regression model for the continuous variables, follicle number, CL size, cleavage rate, and embryo rate. For each categorical variable (pregnancy, breed, follicular response, and cyst presence) the mean and standard deviation were found using a one-way ANOVA. For the effect of multiple variables on the response variable (ie progesterone, pregnancy, and CL size on total follicular number) a fit model was constructed and the parameter estimates for each variable were considered. Not all data was independent because cows were used multiple times. The effects of this were considered in a multivariate analysis and found not to be significantly different from the fit model.

## **Results**

### ***Predicted total number of follicles at time of OPU***

The number of follicles present at the time of OPU does not appear to be affected by the progesterone level alone (Figure 2)( $p=0.65$ ). Progesterone variations due to pregnancy status and CL size were then taken into account. The association between progesterone and follicle number after this adjustment, suggested a slight negative correlation between progesterone and

**Figure 2.** Progesterone versus total follicle number at the time of OPU. No correlation found ( $-0.32 \pm 0.70$  follicles/(ng/ml) progesterone,  $p=0.65$ ).

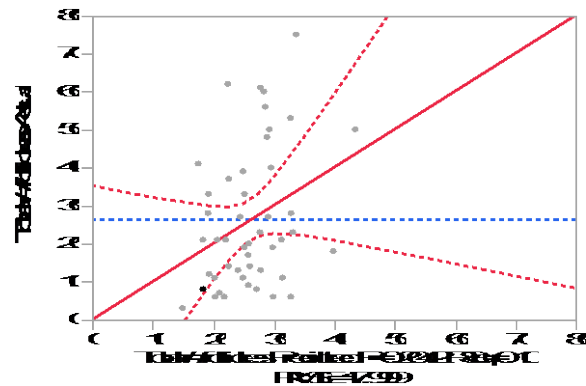


total number of follicles at OPU, though not statistically significant ( $p=0.16$ ).

The larger size of the CL present correlated with an increase in the number of follicles ( $p=0.11$ ). After considering the effects of pregnancy status and progesterone levels (Figure 3), the positive correlation between the CL size and total number of follicles becomes statistically significant ( $0.021 \pm 0.01$ ,  $p=0.04$ ).

It should be noted, however, that follicular response might be indicative of progesterone levels. The responses were categorized as low ( $\leq 10$  follicles), medium (11–25 follicles), and high ( $\geq 26$  follicles). After taking pregnancy and CL size into account, the low responders had a progesterone level  $2.43 \pm 0.96$  ng higher than that of the high responders ( $p=0.01$ ). The progesterone level between medium and high responders was not significantly different ( $p=0.40$ ).

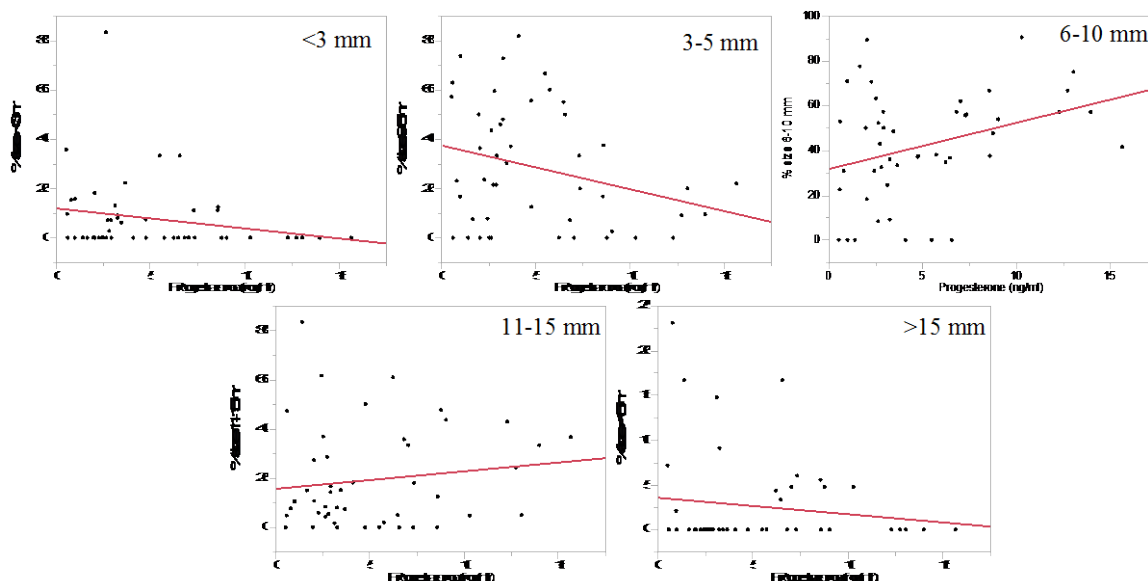
**Figure 3.** Predicted total number of follicles at the time of OPU considering pregnancy status ( $-2.90 \pm 4.54$ ,  $p=0.53$ ), CL size ( $0.02 \pm 0.01$  follicles/ $\text{mm}^2$  CL,  $p=0.04$ ) and progesterone level ( $-1.54 \pm 1.08$  follicles/(ng/ml) progesterone,  $p=0.16$ )



#### *Effects on follicle size at time of OPU*

The trend of the progesterone level and percent of each size of follicle at the day of OPU can be seen in Figure 4. The percent of follicles less than 3 mm did not appear to be correlated with progesterone levels ( $p=0.15$ ), although in cows with a high level of progesterone ( $>10$  ng,  $n=6$ ), there were no small follicles. The percent of follicles 3 to 5 mm was observed to decrease by 1.77 percent for every 1 ng increase in progesterone ( $p=0.05$ ). It appears that cows with a high level of progesterone have a lower percent of small follicles.

**Figure 4.** Progesterone versus percent of follicles in each size category. Percent  $<3$  mm: ( $-0.81 \pm 0.55$ ,  $p=0.15$ ). (3–5:  $-1.77 \pm 0.89$ ,  $p=0.05$ ), Percent 6–10 mm: ( $2.06 \pm 0.87$ ,  $p=0.02$ ), Percent 11–15 mm: ( $0.71 \pm 0.76$ ,  $p=0.36$ ), Percent  $>15$  mm: ( $-0.18 \pm 0.20$ ,  $p=0.36$ )



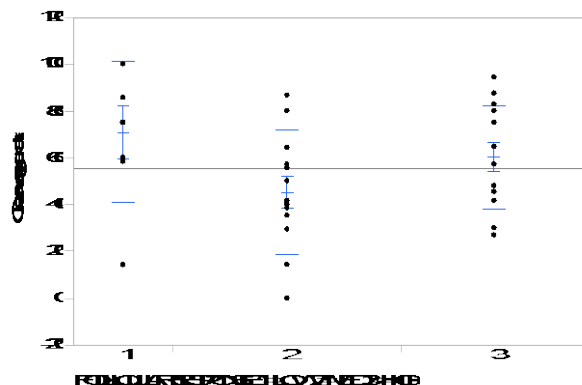
The percent of large follicles present increased as the progesterone levels increased. The percent of follicles 6 to 10 mm increased by 2.06 percent for every 1ng increase in progesterone ( $p=0.02$ ). The increase in percent of follicles 11 to 15 mm was not significant ( $0.71$ ,  $p=0.36$ ). Although most cows did not have a follicle over 15 mm, those that did had a lower progesterone level ( $-0.18$ ,  $p=0.36$ ).

### ***Effects on cleavage rate***

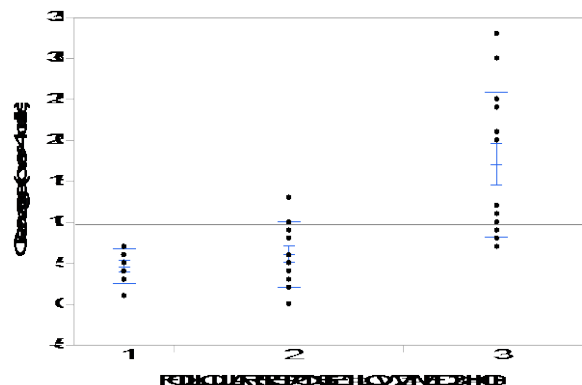
The mean cleavage rate was  $55.41 \pm 27.11$  percent. The cleavage rate does not appear to correlate with progesterone level at the day of OPU ( $p=0.51$ ) (Figure 5), CL size ( $p=0.81$ ), breed ( $p=0.79$ ), cyst presence ( $p=0.77$ ), pregnancy ( $p=0.61$ ), or with total number of follicles ( $p=0.75$ ).

The cleavage rate was highest in the low responders ( $70.48 \pm 30.03$  percent) with rates of  $45.02 \pm 26.81$  and  $60.10 \pm 22.25$  percent in medium and high responders respectively, as seen in Figure 6. However, because of the larger number of oocytes to start with, a high follicular response resulted in  $17.00 \pm 8.92$  more mean cleaved embryos than in medium ( $6.00 \pm 4.03$  embryos) or low ( $4.57 \pm 2.07$  embryos) responders (Figure 7).

**Figure 6. Cleavage rate based on follicular response. Low ( $70.48 \pm 30.03$ ), medium ( $45.02 \pm 26.81$ ), high ( $60.10 \pm 22.25$ )**



**Figure 7. Total number of oocytes cleaved based on follicular response. Low ( $4.57 \pm 2.07$ ), medium ( $6.00 \pm 4.03$ ), high ( $17.00 \pm 8.92$ )**

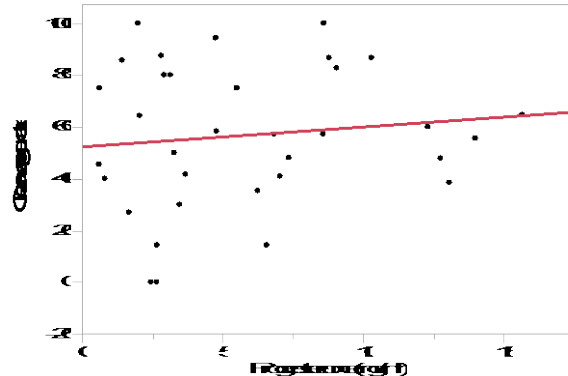


### ***Effects on Embryo Rate***

The mean embryo rate was  $37.14 \pm 23.09$  percent per oocyte. The progesterone did not appear to correlate with embryo rate ( $p=0.96$ ) (Figure 8). Likewise, the effects of pregnancy ( $p=0.95$ ), breed ( $p=0.55$ ), CL size ( $-0.02$ ,  $p=0.17$ ), cyst presence ( $p=0.89$ ), and number of follicles ( $-0.26$ ,  $p=0.17$ ) on embryo rate were insignificant.

Figure 9 shows that the embryo rate was highest in the cows with a low follicular response ( $49.67 \pm 30.17$  percent). Medium and high follicular responses had embryo rates of  $35.01 \pm 22.63$  and  $33.82 \pm 19.30$  percent respectively. The mean number of embryos produced in the high responder group was  $9.94 \pm 5.71$  embryos compared to  $4.26 \pm 2.96$  and  $3.25 \pm 2.49$

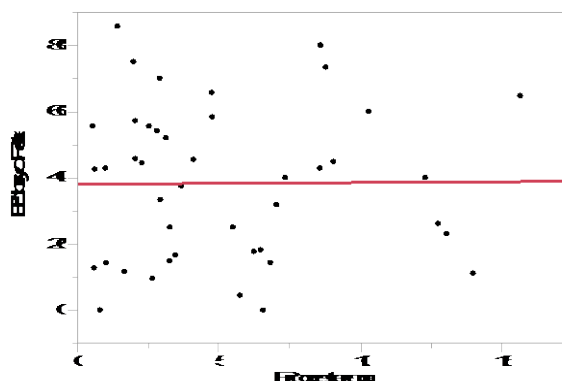
**Figure 5. Progesterone versus cleavage rate. No significant effect ( $0.77 \pm 1.14$  percent/(ng/ml) progesterone,  $p=0.51$ )**



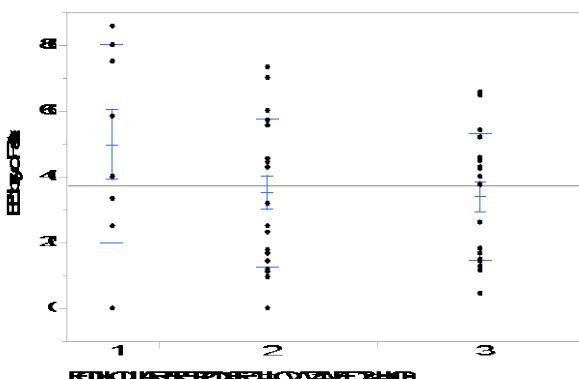
embryos in the medium and low follicular responders respectively (Figure 10). Therefore, having a high response resulted in more embryos per cycle despite the lower cleavage and embryo rates.

Progesterone levels were most strongly influenced by pregnancy ( $p < 0.0001$ ) and the size of the CL present ( $p = 0.01$ ). Measuring the effect of follicle number on progesterone levels after adjusting for the increased progesterone levels due to pregnancy and CL size showed that the effect of follicle number was a small decrease in the progesterone level ( $-0.0332 \pm 0.0204$  ng/ml per follicle), which was not statistically significant ( $p = 0.11$ ). Using the same adjustments, the effect of oocyte number on progesterone showed an insignificant decrease ( $-0.0369 \pm 0.0239$  ng/ml per oocyte,  $p = 0.21$ ). The effect of cleavage rate on progesterone levels showed an insignificant increase ( $0.0135 \pm 0.0116$  ng/ml per percent change in cleavage,  $p = 0.25$ ). Similarly, the embryo rate was also insignificantly increased ( $0.0127 \pm 0.0162$  ng/ml per percent change in cleavage,  $p = 0.43$ ).

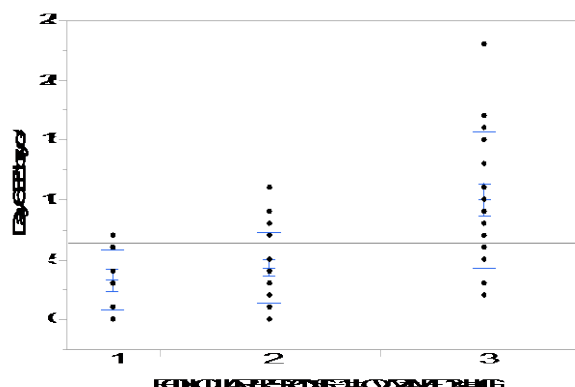
**Figure 8.** Progesterone versus embryo rate. No correlation ( $0.05 \pm 0.89$  percent/(ng/ml) progesterone,  $p = 0.96$ ).



**Figure 9.** Embryo rate based on follicular response. Low ( $49.67 \pm 30.17$ ), medium ( $35.01 \pm 22.63$ ), high ( $33.82 \pm 19.30$ )



**Figure 10.** Total number of embryos based on follicular response. Low ( $3.25 \pm 2.49$ ), medium, ( $4.26 \pm 2.96$ ), high ( $9.94 \pm 5.71$ )



### Laboratory efficiency

Table 1 shows the laboratory efficiency based on the data collected in this study. For the breed breakdowns, it should be noted that the Angus category reflects only the single individual that was used in our study. The mean number of embryos per cycle for the Holsteins was  $3.13 \pm 2.48$  greater than that of the Angus, and  $1.30 \pm 1.69$  than that of the Jerseys. The Jerseys had a mean  $1.83 \pm 2.34$  of more embryos per cycle than the Angus. None of these values were significantly different, with p-values of 0.21, 0.45, and 0.44 respectively.

**Table 1. Laboratory efficiency.**

	Total number of follicles	Number of oocytes	Recovery Rate	Mean cleavage rate*	Total number embryos	Mean Embryo rate**	Mean number Embryos/cycle
Overall	1278	922	72.14%	55.42±27.11 n=36	293	37.14±23.09 n=45	6.36±0.76
Holsteins	550	391	71.09%	57.45±8.77 n=10	112	37.64±6.02 n=15	7.47±1.32
Jerseys	628	443	70.54%	52.95±6.05 n=21	153	34.55±4.76 n=24	6.17±1.05
Angus (660)	61	55	90.16%	61.71±12.40 n=5	26	46.27±9.51 n=6	4.33±2.09

\*not recorded for fertilization attempts, excludes cycles effected by contamination and power failure

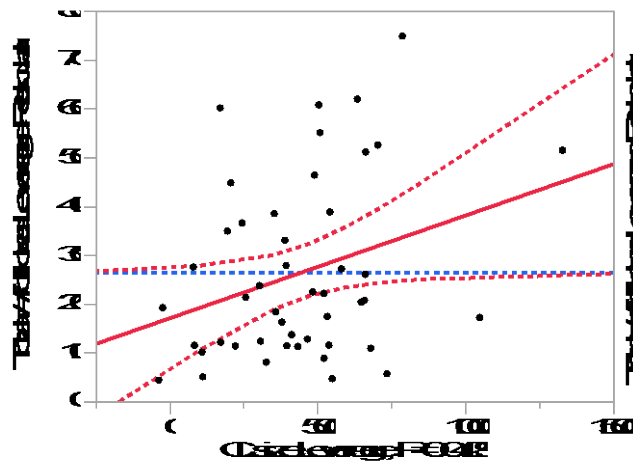
\*\*excludes cycles effected by contamination and power failure

## Discussion

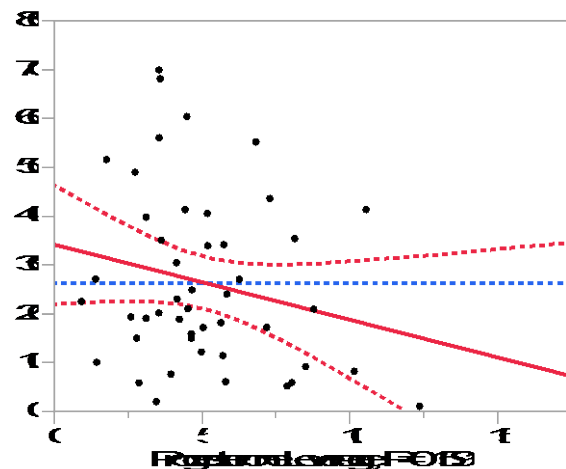
In previous studies on progesterone levels in cows undergoing ovarian stimulation, varying levels of progesterone were attributed to increased number of follicles, enhanced oocyte quality, and an increase in embryo rate [8,9,12,13,15,20,21,24–27]. In this study, cows of various age, parity, breed, and pregnancy status undergoing clinical ovarian stimulation for OPU, had a wide-range in number and competence of their oocytes. Our findings show that using progesterone as an indicator of follicle number, oocyte number, cleavage rate, or embryo rate was ineffective.

Progesterone has previously been thought to effect follicular response due to low progesterone levels (<1ng/ml), which causes increased LH pulsatility [28]. However, even a low amount of progesterone may be enough to effect LH pulsatility, and therefore does not have a significant impact on the follicular response [8,9]. This conclusion was based on only four cycles in cows with <1ng/ml progesterone, which may explain why even though some cows had a low level of progesterone there was no significant change in the number of oocytes.

**Figure 11.** Predicted number of follicles considering the effects of CL size, progesterone, and pregnancy



**Figure 12.** Effects of progesterone on total follicular number considering the effects of CL size, progesterone, and pregnancy





In the cows with the lowest number of follicles, progesterone levels were significantly higher than in those of medium and high responders. The CL size was positively associated with the number of follicles, which conflicts with the findings of EL-Sherry et. al. [29]. EL-Sherry et al. [29] study showed that in cows without a CL present, there were more follicles per cycle and increased estrogen secretion. The positive association of CL size and follicle number may be due to the differing effects of exogenous and endogenous progesterone, or may be due to the increased ovarian size allowing for more room for follicles to develop.

Interestingly, after considering the pregnancy status of the cow, CL size and progesterone appeared to have the inverse relationships to the number of follicles at the time of OPU. As the CL size increased, the number of follicles also increased (Figure 11)( $p=0.04$ ). Conversely, as progesterone level increased, the number of follicles decreased (Figure 12) ( $p=0.16$ ). Investigation of this relationship by varying exogenous progesterone given to cows with a CL present would be interesting area of further research.

Earlier studies have suggested that the size of the follicles present under different levels of progesterone varied and may impact the quality of the oocytes [28]. Studies have found that the percent of total follicles less than 5 mm in diameter was higher in cows with a low level of progesterone. The percent of follicles 6-15 mm was higher in cows with higher levels of progesterone. This difference may have impacts on the quality of the oocytes. This relationship between oocyte competence and size is not linear. In follicles <8mm competence appears to improve with size, while in follicles >12mm competence appears to not be significantly effected [11,12,23].

Progesterone, CL size, breed, cyst presence, or total number of follicles did not affect cleavage and embryo rate. This does not match the results found by both Rivera et. al. [9] and Nasser et. al. [8], where oocyte quality and embryo production was improved in cows give exogenous progesterone. Our results are in agreement with Nivet et. al. [23]. The highest responders did not have the highest cleavage rate. However, our findings showed that having a higher number of follicles at the time of OPU resulted in the highest overall number cleaved and overall number of embryos. Therefore, increasing the follicular response is the best way to increase the number of embryos, even if a lower cleavage rate is observed. Cleavage and embryo rates in our study did not take into account the bull effect, since 27 different bulls were used in the 50 OPU cycles.

In conclusion our study found, progesterone was not a good indicator of a cow's response to ovarian stimulation for OPU. The range in the number of follicles at the time of OPU and the subsequent quality of the oocytes collected showed no strong correlations to the progesterone level at the time of OPU. The size of the CL present was the best indicator of follicular response. This became statistically significant ( $p<0.05$ ) after adjusting for pregnancy and progesterone level. This study concluded that the best way to increase the yield of embryos per cycle was to have a high follicular response. Further studies on the effects of exogenous versus endogenous progesterone may clarify how progesterone changes the response.

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